10.

Tissue Culture and Explantation in Nature: A Review of Certain Experiments and Possibilities.

C. M. Breder, Jr.

New York Aquarium.

Introduction.

The possible methods of operation of organic evolution have for long attracted the speculative faculties of biologists. Phenomena concerned with the general aspects, as well as special phases, have occupied prominent places in biological controversy. In spite of this great discussional activity on the part of students, there is good reason to suppose that many possibilities exist that have not been examined or even imagined. Until all such conceivable methods have been appraised, the extent of the effect of those whose reality is established must remain an open question.

The hypothesis subsequently set forth may serve as an example and undoubtedly numerous others could be developed. All such hypotheses must either be satisfactorily refuted or established in order to develop a thoroughly adequate and fully acceptable evolutionary scheme. The present discussion deals with a very special case, but, as with all such matters, its probable limits of application cannot be easily anticipated at this time.

The argument discussed herewith is thoroughly documented with references to the literature of tissue culture, but as nearly every paper on the subject bears in some way on the present views, only a few have been selected for definite mention. These have been chosen because of their specific application to the points under discussion. See especially the bibliography of Lewis and Lewis (1924) and the *Arch. Exper. Zellforsch*, ed. Erdmann, R. The writer has been given much assistance in assembling the bibliographic data by Dr. J. N. Gowanloch, but is more especially indebted to him for valued criticisms and suggestions.

Factors in Tissue Culture.

An abundant literature, as above noted, has established the fact that animal and plant tissues may be readily cultured when removed from the organism of which they form a part. Such explants, if placed in a suitable medium, will perform their natural functions including those of reproduction. The following brief consideration of pertinent factors will serve to establish the basic data necessary for the purposes of the present discussion.

1. *Cellular immortality:* The small confines of a hanging drop has been sufficient for much tissue culture work, but due to the rapid loss of nutrient value and the accumulation of toxic wastes, such are necessarily limited to short periods unless frequent recourse is made to sub-culturing. With careful renewal of media, explanted cells may be cultured indefinitely and give every indication of being as capable of perpetuating their kind as

any natural organism. Tissue strains from chickens have already reached ages far exceeding the life span of the deriving animal type and show no abatement in vigor. Carrel (1912), Ebeling (1913 and 1922), Baker and Carrel (1926a and b).

2. *Media:* A great variety of solutions is capable of supporting tissue growth and there is a large range of permissible variation in tonicity, pH value, and other important cytologic factors, Lewis and Lewis (1924). As media are usually directly or indirectly imitative of plasma, with or without the addition of nutrient substances, they necessarily tend also to approximate dilute ocean water. It has been shown that various dilutions of sea water itself make satisfactory fluids for cultures in vitro, Lewis (1916), Dederer (1921).

3. *Influence of environment:* Many papers discussing cultures of extirpated cells make mention of changes induced by modifications of the culture media. Other environmental influences are responsible for appropriate modifications in morphology or behavior. Which of these can be considered as genetic, and which the direct impress of environment on each individual cell, is still largely controversial, Uhlenhuth (1915 and 1916). Experiments involving the use of X-rays, however, suggest the former, as might be expected, Strangeways (1924).

4. *Limits of space:* One of the effects of small stagnant bodies of media is outlined under "Cellular immortality." Another is that of excessive bacterial infection. Consequently, tissue culture is usually carried on under sterile conditions in order to prevent the establishment of destructive bacterial colonies. Many kinds of bacteria are preeminently suited to rapid proliferation in a nutrient hanging drop, whereas the explanted cells have no such natural advantage. The same condition is presented in the maintenance of any aquatic organism in a laboratory jar. In any such case the difficulty regarding offensive bacteria is directly proportional to crowding and its attendant effects. It may be readily demonstrated that this is, in part, a function of the quantity of fluid in relation to the organic bulk. It is, therefore, all the more remarkable that numerous students have been able to grow both cells and bacteria in a common drop. Furthermore, they have studied the ensuing interactions and in some cases phagocytosis was observed to be of apparent nutrient value, Johnson (1915), Smyth (1916a and c). The presence of bacteria also apparently acts as a growth-promoting stimulus. Smyth (1916b) wrote of his work, "These results seem to indicate that many bacteria may be utilized by tissue cells as food for growth, or may contain a substance or substances stimulating cell growth or multiplication."

If the preceding four items are considered with mutual reference it becomes apparent that there is no evident reason to prevent the establishment of a culture combining the factors that have already been established separately by a large number of independent investigators. Such a culture would require only some fluid medium of natural occurrence, such as dilute sea water, of sufficient bulk and renewal to prevent an excessive bacterial growth. In other words, the limitations of the tiny bodies of media ordinarily employed, with their consequent favoring of bacteria, must be circumvented. Suggested methods include the following: The fluid medium, in proportion to the organic matter, must be of such a quantity that bacteria have not enough dispersed food to form a dangerously rich culture. This must be below some critical value. In this connection hints are to be found in the maintenance of standing water aquaria in which the food substances must remain below a certain point for similar reasons. An alternative would be a circulation of the medium which might be worked out on a modification of the technique of Burrows (1912). These concepts are obviously derived directly from aquarium practice and it should now be apparent that there is no trenchant difference between the fundamentals of tissue culture and any form of animal or plant husbandry. In all, the

desired organisms are retained in more or less restricted confines and must be protected from enemies, fed, and freed of their own toxic wastes. The smaller the environment in relation to the number of organisms the greater the difficulty in maintaining a persistent culture. In the case under consideration food might have to be introduced manually from time to time in order to avoid nutrient fluids so especially beneficial to bacterial increase.

Experiments such as outlined above are now under way but it has been deemed best to place the above facts and the hypothesis they support on record at this time for the following reasons: The separate elements of the experiment have already been established independently by others. The only service the proposed experiments would perform would be to join certain separate factors. To be of real value it would have to be an operation involving a long period. Negative results might only be an indication of lack of skill on the part of the operator, or some technical difficulty. Others, better equipped than the writer, may be able to contribute more readily to the establishment of proper technique. Some of the problems encountered and the difficulties involved in the experiments thus far performed are discussed subsequently.

Inferences Based on Tissue Culture.

Since all of the conditions discussed are met with in a state of nature, there is good reason to consider the possibility of cultures of this sort arising spontaneously. The natural occurrence of the specific requirements may be considered for comparison with corresponding factors in laboratory cultures.

1. *Sources of materials:* Any event which causes an animal or plant to part with living cells supplies potential material. Such would include destruction by predacious forms, fighting, accidents, physical malfunctioning, such as hemorrhage, and in certain cases normal functioning. Without going into explicit detail it is sufficiently evident that wherever there is any form of life, parts are being continually separated from individuals in relatively immense quantities. Human foetal membranes and menstrual mucosa were successfully cultured by Konrad (1928). Amoebocytes and other cellular elements are normally shed in a living state by many organisms, if not by all.

2. *Media:* Any natural water that is suitable as a culture medium is potentially available for natural explants. For example, brackish sea water has been shown experimentally to be such and is frequently closely similar to plasma. The possibilities would thus vary, both specifically and geographically.

3. *Influences of environment:* Other environmental factors would act favorably or not, according to the nature of the experiment; *e.g.* explants from poikilothermal animals would have little likelihood of being destroyed by the temperature of the environment but homoiothermal ones might require water of approximately body temperature. That explanted cells, even of homoiothermal forms, have a truly remarkable resistance to unfavorable conditions of a most extreme kind has been repeatedly demonstrated. See, for example, Rous and Jones (1916), Nageotte (1927) and Morosow (1928a and b).

4. *Limits of space:* In this regard, the natural culture would be much more advantageously situated than the laboratory drop. It is the same kind of difference that obtains between a small aquarium and a body of open water. In a state of nature the food is automatically supplied and toxic wastes are either washed away or rendered innocuous.

5. *Enemies:* Instead of being protected from enemies, natural explants would have to take the chance of any organism invading a new environment. Other things being equal, the dangers from bacteria would be vastly less than in a laboratory culture. This limits successful establishment to rea-

sonably "clean" environments and rules out those that are characterized by a large amount of decomposition. In some environments, consisting of long-standing organic equilibria, bacteria seem to be at a disadvantage because of the presence of a lysisime or bacteriophage. Such conditions not infrequently may be found in standing aquaria, Breder (1931). In this connection it is notable that the cells in their original locations within an animal body are not in a sterile environment. The sterility of a culture medium is chiefly a concession for the enforced disadvantages of the cramped quarters, comparable to the omission of the natural enemies of fishes in an aquarium. Considering such explants as invading organisms it should be borne in mind that they would usually be reinforced continually by similar cells from the original source. An example would be the case of a predacious animal feeding chiefly on a single food animal (a type of specificity of frequent natural occurrence) with the continued escape of its tissues on being crushed. Since embryological material is generally more suitable for tissue culture, it may be noted that gravid females falling prey or the robbing of nests would abundantly supply much material. A hypothetical case is given below for illustrative purposes.

The arena might be a newly formed tropical swamp where the sea has inundated a low fresh water bog, depressing the quantity of its micro-fauna (protozoans, bacteria, et cetera), and in which the ocean water fauna (somewhat dilute) has not yet thoroughly established itself, but in which currents have washed various areas clean. Crocodiles could be feeding rapaciously on small animals, water fowl, et cetera, and so release—in some cases as single cells—the various constituents of the blood and in a less quantity epithelium, foetal material, et cetera.

It is experimentally demonstrable that an abundance of such explants are actually being continually released wherever there is a struggle for existence, that suitable natural media are clearly of worldwide occurrence and that the potency of the chief primary enemies, bacteria, is generally inversely proportional to the size of the fluid environment. Since the first appearance of the earliest metazoans the proper constellation of factors could easily have occurred many times. With this as a working hypothesis, we may consider some of the more apparent philosophical implications. Either such explants are capable of continued life, as is indicated by long-time cultures, or they are not. If the latter is true they would all eventually die off, but in the interim would exist to confound protozoan systematics. Attention need hardly be called to the striking similarity in morphology and behavior between free metazoan cells and protozoa, *e.g. Amoeba* and phagocytes, Hogue (1922), or ciliates and the singly freed cells of echinoderm eggs, Jenkinson (1909). Since numerous tissue culturists have shown that various environmental factors influence the morphology and behavior of their explants, natural cultures would certainly show comparable changes and probably bear little resemblance to their deriving tissues.

It has been shown that protozoans do not necessarily require fertilization to maintain vitality in subsequent generations. In eleven thousand generations of *Paramecium,* Woodruff (1926) found perfectly normal individuals produced by binary fission, but he did find some activity within single individuals involving a rearrangement of nuclear material for which he created the term endomixis, Woodruff and Erdmann (1914), Woodruff (1925), Calkins (1926). Behavior of nuclei of cultured tissues strangely resembling endomixis has been repeatedly observed, Holmes (1914), Macklin (1916) and Dederer (1921). There is no reason to believe that there is any fundamental functional difference between such acts and the observations of Woodruff. With these facts at hand it is quite conceivable that explanted cells under proper environmental stimulus might even show other than binary fission. It would be interesting to know whether the nuclear adjustments in tissue cultures are stimulated by explanting, or

whether they represent normal activity. If the latter is true the connection of the possible loss of such activity with senility and the relatively poor behavior of adult material in vitro is suggested. It is to be noted in this connection that while explanted cells usually retain their individual cell characteristics they frequently show dedifferentiation pointing to a less specialized and somewhat embryonic condition, Lewis (1920), Fisher (1922). These effects are clearly involved in the lack of endocrine and other control. For example, there is evidence that plasma from old animals checks growth in vitro, Baker and Carrel (1926 a and b).

Contemplating the other alternative, that natural explants are capable of survival, there is nothing remaining to separate them from the protozoa, and as they have been experimentally shown to be reactive to environmental stimulus there is no reason to suppose that they lack the evolutionary possibilities attributed to other forms of life. With such an assumption we should have "protozoa" derived directly from metazoa but charged with specific potentialities of various sorts dependent on the functions of their immediate ancestors as parts of a metozoan unit. Geneticists may object to this as a possibility on a basis of theory involving the continuity of germ plasma as distinct from somatic structure. However, there appears to be no theory or genetical experiments that have any bearing on the matter and the distinction referred to cannot be extended to the present case, for with self-sustaining and reproducing cells entirely free from the originating metazoan unit they at once may be considered germinal and somatic in every sense that a conventional protozoan is so considered.

A view set forth by Marchand (1935) would seem somewhat to approach the present in that it attempts to derive solitary coelenterates from colonial forms. Actually this view has little in common with the present, since the argument runs to the effect that evolution from "single cells" to complex forms was through colonial forms which subsequently separated, rather than direct. Be that as it may, the present hypothesis concerns itself solely with the ability of organic fragments to survive and to evolve.

The consequences of the present view would be to upset the unity of phylogeny and if shown tenable would introduce complications in a number of disciplines. Just how far this might be carried is of course very uncertain, but it could conceivably account for the lack of convergence in geologic time of certain phyla. It is difficult to imagine the tracing of a major phylogenetic tree with such a condition obtaining. As all the conventional phyla extend a great distance in geologic time it would follow that an origin from such a source could have occurred only in the remote past, except possibly considering the protozoa as a mixed phylum. This would tend to discourage the entire thought, were it not for the fact that there are a large number of organisms that have no convincingly evident affinities and have been buffeted about in a vain effort to find a place for them, as is witnessed by any zoological text book with its numerous "appendices" or "incertae sedis." For example, Parker and Haswell (1910) list eleven phyla and to these are added seven "appendix" forms. Without attempting to press the speculation too far it is clear that this condition obviates any necessity to allocate such an origin of forms to any particular geologic period.

A consideration of the general geographical requirements of derivation might be somewhat as follows: *Tropical,* homoiothermal vertebrates; *any climate,* poikilothermal vertebrates, invertebrates, plants.

Coupled with this list must go such factors of environment as suitable chemical quality of the water, a proper osmotic pressure, a sufficient freedom from enemies, et cetera (*i.e.* a sufficiently suitable environment).

As it has been shown that a variety of transplants is possible from one organism to another, for example blood transfusions, glandular transplants, plant and animal grafting, et cetera, on the basis of the preceding, occasionally natural transplants might well originate. The remarkable production

of complex plant monsters, Winkler (1914), and the composite *Hydras* of Issayev (1924), both show the ability of very different cells to live side by side. If recent explants are ingested or lodged in surface wounds the possibility of their survival arises. Certain carcinoma cells similar to their associates except in growth rate come to mind, including even the possibility of self-infection. It is noteworthy that malignant growths are most common at first near the surface or in the alimentary or other tract open to the exterior. Thus, a return cycle further complicating conditions would have bearing on a large number of academic and practical matters. At least it is certain the symbiotic relationships must have arisen in some such accidental manner. *Hydra viridis*, *Convoluta roscoffensis*, the lichens, or even man and his intestinal flora may serve as examples of various kinds of such associations. Buchsbaum and Buchsbaum (1934) produced what they considered an artificial symbiosis between tissue culture cells and the algae, *Chlorella*.

Passing beyond the limits of the indicated inferences of this hypothesis, we might even imagine bacteria to be mitochondria released in a similar manner. This would be inverse to the idea set forth by various students, most recently Wallin (1927), which view is objected to by Cowdry (1924). Viruses might be thought of as also being involved and derived from the fluids of ruptured cells. Schultz (1930) gives a general view of the behavior of viruses very suggestive in this connection. See also the discussion by Riddle (1936). It is recognized that the above is pure speculation and it is mentioned merely as indicative of the lines of thought engendered by a consideration of certain facts in the technique of tissue culture.

Difficulties of Experimental Procedure.

The experimental establishment of explanted tissues in relatively "wild" environments would place the entire suggestion on a relatively firm foundation. As previously noted there are both experimental, and, in the case of the writer, personal difficulties involved. Nevertheless, a considerable amount of experimentation was undertaken. For very substantial aid in this the writer is indebted to Dr. R. F. Nigrelli who labored with most of the actual physical material.

The following discussion of the results of this work is introduced chiefly to point out that in addition to ordinary experimental difficulties there are likewise theoretical obstacles to the establishment or destruction of the present hypothesis by experimental means. The discussion of certain experiments may serve to demonstrate the point.

The leucocytes of invertebrates were thought of as likely material for such experimentation. For example, those of the common oyster normally invade the mantle cavity and are frequently voided into the surrounding water, especially under slightly suffocating conditions, Orton (1924), Young (1928). This phenomenon, diapedeses, is apparently common to a variety of animals. Leucocytes so voided by the American species, *Ostrea virginica*, were found to live for as long as six days in a laboratory dish with no attention whatsoever. Breder and Nigrelli (1933), while those of the European oyster, *O. edulis*, lived for Orton up to four days. Such, then, would seem to be ideal material.

However, it so happens that *O. virginica* is infested with amoebic parasites (or commensals?), *Vahlkampfia calkinsi*, or *V. patuxent* or both. These are usually found in the intestinal tract, are voided with the faeces, and bear a strong superficial resemblance to the leucocytes. The latter likewise invade the intestinal tract in the course of their functional activity. The describer of these parasites, Hogue (1915, 1921 and 1922), was only able to distinguish them from the leucocytes after fixation, when with suitable staining the nuclear material was found to be differently arranged. Breder

and Nigrelli (1933) found in their dishes that in addition to parasites voided in the faeces, and leucocytes voided from the gill chamber, they also had a free living *Amoeba* of the *limax* type that normally lives on the exterior of the shell. This latter form caused no confusion, however. In agreement with Hogue they found (unpublished) that pure cultures of leucocytes withdrawn from the heart would not survive on agar plates for any great length of time, but that the parasites from the intestine could be so cultured. Thus it follows that the differentiation of leucocytes from parasites is dependent on (1) arrangement of nuclear material, for which the material must be killed and stained, and (2) ability to grow on agar plates.

Leucocytes from the heart will not grow on plates but material from the intestine will. Both parasites and leucocytes (as based on stained material) are found there, but on old agar plates only the "parasite" type of nucleus is found. It seems to the writer that there is just an even chance that these "parasites" may be one phase of the normal oyster leucocyte, especially since they always seem to be present. If they could be shown to be a phase or type of leucocyte occurring only in the gut, which has the possibility of exterior survival, this could be used for considerable support of the hypothesis. As it stands—parasite, commensal or leucocyte—it is clear that any point of view can be argued and experiments may prove one or the other, depending on the experimenter's bias, with no present hope of experimentally further separating the material.

Giving this line up, earthworms were examined, since they void amoeboid cells with their casts. Without going into details it may be stated that earthworms also harbor amoeboid parasites and a similar block to this line checked an experiment that seemingly held promise and made us wonder if all commensal or innocuous amoebic parasites were simply transformed leucocytes.

The application of micro-dissection to the problem involves a further philosophical consideration but points the way to the types of material that may hold some hope for experimental verification. If certain types of animals are disassociated the pieces will reunite to re-form the originals. Sponges, perhaps, represent the best known case of this sort. *Hydra* will also show this phenomenon, Papenfuss (1934), but under certain conditions will not. The fate of the individual cells under such conditions is not yet certain. Obviously, freed cells that reunite to construct organisms cannot be expected to be of much value for this kind of experiment. On the other hand highly specialized and protected cellular elements could hardly be expected to survive without the complex of conditions under which they normally exist. Consequently the type of tissue that presumably must be sought after in this connection is something sufficiently unspecialized as to be able to survive in a new environment and still without the ability to reconstruct an individual animal with its fellows. In this connection it may be pointed out that it is sometimes surprising to note what ordinarily well protected tissues may do in the way of survival under exposed conditions. Nigrelli and Breder (1935) describe a prolapsed fish intestine, which while fed with body juices was exposed to the ordinary standing water of an aquarium. This pendant piece proliferated for several months and was finally killed for study.

Conclusion.

The hypothesis that animal and plant cells when dislodged from their original locations *in situ* by natural causes may continue living independently as distinct organic units rests on a large number of concrete experimental demonstrations by independent investigators. These contributions were all made with reference to special and distinctly diverse problems not in the least connected with the present integrated interpretation of them.

That they are adequate and pertinent to this hypothesis can be sustained by reference to the total literature of tissue culture and the absence therein of any contrary findings. Experimental verification, however, must wait on the development of a more satisfactory approach than is now available. The author, at least, has thus far been unable to devise a practicable experiment, the results of which can be interpreted in but one way. The continued presence of this duality of possible interpretation stands as an impediment to experimental analysis of the problem. The conception of a critical experiment must be realized before further progress can be expected. That tissue culture has been possible and that a large variety of cells have been grown and have perpetuated themselves for long periods in a considerable variety of environments may be taken as good presumptive evidence in favor of this hypothesis.

Bibliography.

Baker, L. E. and Carrel, A. 1926A. Au Sujet du Pouvoir inhibiteur du Serum pendant la Vieillesse. *Compt. Rend. Soc. Biol.*, 95, pp. 958-960.

..... 1926B. Action on fibroblasts of the protein fraction of embryonic tissue extract. *Journ. Exp. Med.*, 44, pp. 387-407.

Breder, C. M. Jr. 1931. On organic equilibria in aquaria. [Abstract.] *Copeia*, (2), p. 66.

Breder, C.M. Jr. and Nigrelli, R. F. 1933. *Lamellibranch* leucocytes as living material for class-room demonstration. *Science*, 78, (2015), p. 128.

Buchsbaum, R. and Buchsbaum, M. 1934. An artificial symbiosis. *Science*, 80, (2079), pp. 408-409.

Burrows, M. T. 1912. A method of furnishing a continuous supply of new medium to a tissue culture in vitro. *Anat. Rec.*, 6, pp. 141-144.

Calkins, G. N. 1926. The Biology of the Protoza. Lea and Febriger, *Philadelphia*, pp. 1-623.

Carrel, A. 1912. On the permanent life of tissues outside of the organism. *Journ. Exp. Med.*, 15, pp. 516-528.

Cowdry, E. V. 1924. General Cytology. University Chicago Press, *Chicago*, pp. 1-754.

Dederer, P. H. 1921. The behavior of cells in tissue cultures of *Fundulus heteroclitus*, with special reference to the ectoderm. *Biol. Bull.*, 41, (4), pp. 221-240.

Ebeling, A. H. 1913. The permanent life of connective tissue outside of the organism. *Journ. Exp. Med.*, 17, pp. 273-285.
..... 1922. A ten year old strain of fibroblasts. *Journ Exp. Med.*, 35, pp. 755-759.

Fischer, A. 1922. A pure strain of cartilage cells in vitro. *Journ. Exp. Med.*, 36, pp. 379-384.

Hogue, M. J. 1915. Studies in the life history of an amoeba of the Limax group, *Vahlkampfia calkinsi. Arch. f. Protist.*, 35, (2), pp. 154-163.

..... 1921. Studies on the life history of *Vahlkampfia patuxent* n. sp. parasite on the oyster, with experiments regarding its pathogenicity. *Am. Journ. Hyg.*, 1, pp. 321-345.

..... 1922. A comparison of an amoeba, *Vahlkampfia patuxent* with tissue culture cells. *Journ. Exp. Zool.*, 35, (1), pp. 1-11.

Holmes, S. J. 1914. The behavior of the epidermis of amphibians when cultivated outside of the body. *Journ. Exp. Zool.*, 17, pp. 281-295.

Issayev, V. 1924. Researches on animal chimeras. *Journ. Genet.*, 14 (3), pp. 273-351.

JENKINSON, J. W. 1909. Experimental Embryology. Clarendon Press, *Oxford.* pp. 1-341.

JOHNSON, J. C. 1915. The cultivation of tissues from amphibians. Univ. Calif. Pub. in *Zool.*, 16, pp. 55-62.

KONRAD, H. 1928. Lebens—und Wachstumsbeobachtungen an Menschlichen Geweben und Geschwulsten im Explantationsversuche und ihre Bedeutung für klinische Fragen. *Arch. Gynakol.*, 134, (2) pp. 250-309.

LEWIS, M. R. 1916. Sea water as a medium for tissue cultures. *Anat. Rec.*, 10, pp. 287-299.

..... 1920. Muscular contraction in tissue cultures. Contrib. Embryol., 9, Carnegie Inst. Wash. Pub. 272, pp. 191-212.

LEWIS, W. H. and LEWIS, M. R. 1924. In Cowdry, E. V. General Cytology, Univ. Chicago Press., *Chicago,* pp. 383-447.

MACKLIN, C. C. 1916. Amitosis in cells growing in vitro. *Biol. Bull.*, 30, pp. 445-466.

MARCHAND, W. 1935. Remarks on the evolution of animal phyla. [Abstract.] *Anat. Rec.*, 61, (2), pp. 3-4.

MOROSOW, B. D. 1928A. Explantationsversuche mit getrockneten Amphibienherzen. *Arch. Exp. Zellforsch,* 7, (2), pp. 213-220.

..... 1928B. Explantationsversuche an getrockneten und wiederbelebten Herzen der Menschen—und Hubnerembryonem. *Munchener Med. Wochenschrift,* 75, (40), p. 1713.

NAGEOTTE, J. 1927. Über die Überpflanzung Von Abgetoteten Bindegewebsstucken Erwiderung an Fr. Weidenreich und A. Busacca. *Virchow's Arch. fur Pathologische anatomie und Physiologie,* 263, (1), pp. 69-88.

NIGRELLI, R. F. and BREDER, C. M. JR. 1935. Histological changes in the prolapsed intestine of a fish, *Mollienisia latipinna* Le Sueur. *Copeia,* (3), pp. 68-72.

ORTON, J. H. 1924. An account of investigation into the cause or causes of the unusual mortality among oysters in English oyster beds during 1920 and 1921. *Fisheries Invest.* Ser. II, 6, (3).

PAPENFUSS, E. J. 1934. Reunition of pieces in Hydra, with special reference to the role of the three layers and to the fate of differentiated parts. *Biol. Bull.*, 67, pp. 223-243.

PARKER, J. T. and HASWELL, W. A., 1910. A textbook of Zoology. 2nd ed. Macmillan Co., *London.* 2 Vol.

RIDDLE, O. 1936. The Confusion of Tongues. *Science,* 83 (2142), pp. 41-45.

ROUS, P. and JONES, F. S. 1916. A method for obtaining suspensions of living cells from fixed tissues and for the plating out of individual cells. *Journ. Exp. Med.*, 23, pp. 549-555.

SCHULTZ, E. W. 1930. The ultrascopic viruses from the biological standpoint. *Scientific Monthly,* November, pp. 422-433.

SMYTH, H. F. 1916A. The reactions between bacteria and animal tissues under conditions of artificial cultivation. II. Bactericidal action in tissue cultures. *Journ. Exp. Med.*, 23, pp. 265-274.

..... 1916B. The reactions between bacteria and animal tissues under conditions of artificial cultivation. III. The action of bacterial vacine on tissue culture in vitro. *Journ. Exped. Med.*, 23, pp. 275-281.

..... 1916C. The reactions between bacteria and animal tissues under conditions of artificial cultivation. IV. The cultivation of tubercle bacilli with animal tissues in vitro. *Journ. Exp. Med.*, 23, pp. 283-291.

STRANGEWAYS, T. S. P. 1924. Tissue culture in relation to growth and differentiation. W. Heffer & Sons, *Cambridge*. pp. 1-50.

UHLENHUTH, E. 1915. The form of the epithelial cell in cultures of frog skin and its relation to the consistency of the medium. *Jour. Exp. Med.*, 22, pp. 76-104.

..... 1916. Changes in pigment epithelium cells and iris pigment cells of *Rana pipiens* induced by changes in environmental conditions. *Journ. Exp. Med.*, 24, pp. 689-699.

WALLIN, I. E. 1927. Symbionticism and the origin of species. Williams and Wilkins, *Baltimore*, pp. 1-171.

WINKLER, H. 1914. Chimarenforschung als methodi der experimentelle Biologie. *Sitzungs Berichta Physik Med. Gesselschaft Jahrgeng* 1913 (8) pp. 113-119.

WOODRUFF, L. L. 1925. The physiological significance of conjugation and endomixis in the infusoria. *Amer. Nat.* 59, pp. 225-249.

..... 1926. Eleven thousand generations of *Paramecium. Quart. Rev. Biol.*, 1 (3), pp. 436-438.

WOODRUFF, L. L. and ERDMANN R. 1914. A normal periodic reorganization process without cell fusion in *Paramecium. Journ. Exp. Zool.*, 17, (4), pp. 425-502.

YONGE, C. M. 1928. The absorption of glucose by *Ostrea edulis. Journ. Mar, Biol. Assoc.*, 15 (N.S.), pp. 643-653.